Critical role of Arg^{433} in rat transketolase activity as probed by site-directed mutagenesis

Yunjo SOH, Byoung J. SONG[1], Jiingjau JENG and Abraham T. KALLARAKAL

Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, 12501 Washington Avenue, Rockville, MD 20852, U.S.A.

It has been shown that one arginine per monomer at an unknown position is essential for enzyme activity of the homodimeric transketolase (TK) [Kremer, Egan and Sable (1980) J. Biol. Chem. **255**, 2405–2410]. To identify the critical arginine, four highly conserved arginine residues of rat TK (Arg^{102}, Arg^{350}, Arg^{433} and Arg^{506}) were replaced with alanine by site-directed mutagenesis. Wild-type and mutant TK proteins were produced in *Escherichia coli* and characterized. The Arg^{102} → Ala mutant exhibited similar catalytic activity to the wild-type enzyme, whereas Arg^{350} → Ala, Arg^{506} → Ala and Arg^{433} → Ala mutants exhibited 36.7, 37.0 and 6.1 % of the wild-type activity respectively. Three recombinant proteins (wild-type, Arg^{350} → Ala and Arg^{433} → Ala) were purified to apparent homogeneity using Ni^{2+}-affinity chromatography and further characterized. All these proteins were able to form homodimers (148 kDa), as shown by immunoblot analysis subsequent to non-denaturing gel electrophoresis. The Arg^{433} → Ala mutant protein was less stable than the wild-type and Arg^{350} → Ala proteins at 55 °C. Kinetic analyses revealed that both V_{max} and K_m values were markedly affected in the Arg^{433} → Ala mutant. The K_m values for two substrates xylulose 5-phosphate and ribose 5-phosphate were 11.5- and 24.3-fold higher respectively. The k_{cat}/K_m values of the Arg^{433} → Ala mutant for the two substrates were less than 1 % of those of the wild-type protein. Molecular modelling of the rat TK revealed that Arg^{433} of one monomer has three potential hydrogen-bond interactions with the catalytically important highly conserved loop of the other monomer. Thus, our biochemical analyses and modelling data suggest the critical role of the previously uncharacterized Arg^{433} in TK activity.

INTRODUCTION

Transketolase (TK) (EC 2.2.1.1) is a cytosolic enzyme which transfers a two-carbon unit among various carbohydrates. This enzyme reversibly interconnects the glycolytic and the pentose monophosphate shunt pathways, providing pentose sugars which are essential building blocks for various biochemical reactions [1,2]. It has been well established that TK is active as a homodimer and requires the cofactor thiamin pyrophosphate (TPP) and divalent cations such as Mg^{2+} or Ca^{2+} for its activity [3]. During TK-mediated catalysis, a proton from the C-2 atom of the thiazolium ring of bound TPP is abstracted by His^{481} in the highly conserved loop (amino acids 468–483 of yeast TK, equivalent to amino acids 447–462 of rat TK) [4]. As a result, the C-2 atom of TPP is readily converted into the corresponding carbanion [2,4,5]. This reactive carbanion intermediate then interacts with a donor ketose substrate, xylulose 5-phosphate, to form α,β-dihydroxyethyl TPP (+a two carbon unit) before an acceptor substrate, ribose 5-phosphate, receives the two carbon unit from the α,β-dihydroxyethyl TPP [2,4,5]. TPP was also shown to be absolutely required for the subunit interaction, regardless of protein concentration [6]. Reductions in TK activities have been observed in certain disease states, such as Wernicke–Korsakoff syndrome [7,8], alcoholism [9] and Alzheimer's disease [10,11]. The decreased TK activity was not readily recovered even after restoring normal thiamin levels [12]. The reduced activity could be due to a different affinity (up to a 10-fold increase in K_m value) for TPP [9,13].

Using two arginine-specific reagents, phenylglyoxal and 2,3-butanedione, Kremer et al. [14] demonstrated that two arginine residues per homodimeric holoenzyme (one arginine per monomer) were essential for TK activity. However, it is still unknown which arginine is critical for TK activity. We therefore examined the functional role of four arginines of the rat enzyme (Arg^{102}, Arg^{350}, Arg^{433} and Arg^{506}) which are highly conserved among TKs in various species [15–20]. Each arginine residue was replaced with alanine by site-directed mutagenesis. Three recombinant rat TK proteins (the wild-type and Arg^{350} → Ala and Arg^{433} → Ala mutants) were produced in *E. coli*, purified to near homogeneity and subjected to biochemical and biophysical characterization. During the course of our characterization of TK mutants, Nilsson et al. [21] reported that Arg^{359} and Arg^{528} of yeast TK (equivalent to Arg^{350} and Arg^{506} of rat TK) are involved in substrate binding. Our biochemical data and structural analysis by molecular modelling indicate that a previously uncharacterized Arg^{433} of rat TK is crucial for the TK activity, as it maintains the inter-subunit interactions between this arginine on one monomer and the highly conserved loop on the other monomer.

EXPERIMENTAL

Reagents

BL21(DE3) competent bacterial cells and plasmids [TA cloning vector and pET28a(+) plasmid] were purchased from Novagen (Madison, WI, U.S.A.). Taq polymerase was obtained from Promega. [γ-^{35}S]dADP was purchased from DuPont–New England Nuclear (Boston, MA, U.S.A). Protein molecular mass markers for denaturing and non-denaturing gels were obtained from Gibco-BRL (Gaithersberg, MD, U.S.A.) and Pharmacia

Abbreviations used: TK, transketolase; TPP, thiamin pyrophosphate.

[1] To whom correspondence should be addressed (e-mail bs7t@nih.gov).

Table 1 Primer pairs used in site-directed mutagenesis

Underlined oligonucleotides represent the base substitutions for mutagenesis. DNA amplification by two cycles of PCR was performed as described in the Experimental section [22].

Sense oligomer (5′-end)	
Forward	5′-dATA CAT ATG GAT CCA GTG AGA CAG ATC CAG-3′
Anti-sense oligomer (3′-end)	
Reverse	5′-dGAA TTC TCC TTC CTA GCC CTT GGT GAC AAG-3′
$Arg^{102} \rightarrow Ala$	
Forward	5′-dAAC CCT CAC AAT GAT GCC TTT GTG CTC TCC-3′
Reverse	5′-dGGA GAG CAC AAA GGC ATC ATT GTG AGG GTT-3′
$Arg^{350} \rightarrow Ala$	
Forward	5′-dGAC AAG ATA GCC ACA GCC AAA GCC TAG GGA T-3′
Reverse	5′-dA TCC ATA GGC TTT GGC TGT GGC TAT CTT GTC-3′
$Arg^{433} \rightarrow Ala$	
Forward	5′-dTTC GAC CAG ATC GCC ATG GCC GCC ATC TCC-3′
Reverse	5′-dGGA GAT GGC GGC CAT GGC GAT CTG GTC GAA-3′
$Arg^{506} \rightarrow Ala$	
Forward	5′-dATT CGG ACC AGC GCC CCA GAA AAT GCC ATT-3′
Reverse	5′-dAAT GGC ATT TTC TGG GGC GCT GGT CCG AAT-3′

(Piscataway, NJ, U.S.A.) respectively. All other reagents were from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Generation of PCR products and site-directed mutagenesis

Various oligodeoxynucleotides, as listed in Table 1, were synthesized with a nucleotide synthesizer (Applied Biosystems model 394), using the procedure recommended by the manufacturer. All PCRs were performed using a DNA thermal cycler 9600 (Perkin-Elmer Cetus). Amplification of DNA fragments was achieved by adding 5 ng of the template DNA, 20 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM each of dNTPs, 0.1 mg of each set of oligonucleotide primers and 1 unit of Taq polymerase (final volume 50 μl). Each sample was subjected to 25 cycles of amplification consisting of denaturation (1 min, 95 °C), annealing (2 min, 52 °C) and extension (2 min, 72 °C).

Site-directed mutagenesis was performed by an overlap extension method which includes two rounds of PCR, as described [22]. In the first run, two DNA fragments encompassing the entire protein coding region of the rat TK cDNA sequence [20] were amplified. Each reaction contains one flanking primer that hybridized at one end of the TK cDNA sequence (5′-end or antisense 3′-end primer) and one mutagenic sequence (forward or reverse as listed in Table 1). The two DNA fragments generated in the first PCR were gel purified and used as templates as well as primers in the subsequent extension reaction to encompass the entire protein coding region of the rat TK. Each of the mutant TK cDNA sequences was cloned into a TA cloning vector. The *Nde*I–*Eco*RI-cleaved fragments of the resulting plasmid containing the TK cDNA sequences were subcloned into the pET28a(+) expression vectors with the His-Tag sequence of six histidine residues located at the 5′-end of the TK cDNA sequences. The resulting plasmids were transformed into *E. coli* BL21(DE3) competent cells according to the manufacturer's protocol. Correct nucleotide sequences for the wild-type and mutant TKs were confirmed by nucleotide sequencing using the dideoxy chain-termination method [20].

Expression and purification of recombinant TK proteins in *E. coli*

E. coli BL21(DE3) cells harbouring various pET28a(+)-TK plasmids were grown in Luria–Bertani medium (1 liter) containing kanamycin (30 μg/ml). Bacterial cells were grown at 37 °C to an absorbance of 0.4 at 600 nm and then induced with 0.4 mM isopropyl 1-thiogalactopyranoside for another 4 h before harvesting by centrifugation at 3000 ***g*** for 10 min. Cell pellets were resuspended in 20 ml of a buffer containing 20 mM Tris/HCl (pH 7.9), 500 mM NaCl, 5 mM imidazole, 1 mg/ml leupeptin, 100 μM PMSF and 20 % (v/v) glycerol. Resuspended bacterial cells were then subjected to two rounds of sonication (output control at 6, Branson Sonifier 250) for 30 s on ice. After centrifugation at 9000 ***g*** for 20 min, the resulting supernatant from each sample was applied to the Ni^{2+}-affinity column (2.5 ml of His-bind resin; Novagen). The column was washed with 16 vol. of washing buffer (60 mM imidazole/500 mM NaCl/20 mM Tris/HCl, pH 7.9) and various rat TK proteins were eluted with 6 vol. of elution buffer (1 M imidazole/500 mM NaCl/20 mM Tris/HCl, pH 7.9).

Determination of TK activity and kinetic parameters

The catalytic activities of the recombinant wild-type and mutant TK proteins in the soluble fractions of *E. coli* were determined by monitoring the production of sedoheptulose 7-phosphate, as described by Takeuchi et al. [23]. TK activity was calculated by subtracting the activity of the bacteria cells lacking the TK cDNA insert. A coupling assay method [24] was used for the purified recombinant TK proteins, with the following modifications. Activity was measured in a 1 ml reaction mixture containing 10 mM Tris/HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM ribose 5-phosphate, 1 mM xylulose 5-phosphate, 2 units of glycerol 3-phosphate dehydrogenase, 2 units of triose-phosphate isomerase, 0.1 mM NADH and 0.2 mM TPP at 25 °C. One unit of TK was defined as that required for the oxidation of 1 μmol of $NADH \cdot min^{-1} \cdot (mg\ of\ protein)^{-1}$. Kinetic parameters were determined from measurements of the initial rates of NAD^+ production from NADH at different concentrations (ranging from 0.08 μM to 20 mM) of xylulose 5-phosphate or ribose 5-phosphate in the presence of 6 or 10 mM of the other pairing substrate. The reaction was initiated by the addition of the purified TK (25 μM of wild-type or $Arg^{433} \rightarrow Ala$ mutant per reaction). The apparent V_{max} and K_m values were calculated using a hyperbolic non-linear regression analysis program (hyper.exe) available on the internet (http://www.liv.ac.uk/~jse/software.html).

Molecular modelling

Homology structural modelling of rat TK was carried out using the modelling program LOOK (Molecular Applications Group, Palo Alto, CA, U.S.A.). The coordinates for the crystal structure of the yeast TK homodimer [21] were used as the template for homology modelling. The protein sequence of the rat TK was initially aligned with that of yeast TK using the program LOOK which utilizes the Needleman–Wunch algorithm. A three-dimensional structure of rat TK homodimer was generated using this alignment and the modelling program Segmod [25]. Similarly, three-dimensional structures were generated for the mutants $Arg^{433} \rightarrow Lys$ and $Arg^{433} \rightarrow Ala$.

Non-denaturing gel electrophoresis

Soluble fractions from *E. coli* lysates harbouring the wild-type rat TK cDNA or mutants ($Arg^{350} \rightarrow Ala$ and $Arg^{433} \rightarrow Ala$) were subjected to non-denaturing gel electrophoresis for 12 h at a constant 25 V at 4 °C. The proteins were transferred to a nitrocellulose membrane and subjected to immunoblot analysis

using the polyclonal antibody against rat TK. Other materials and methods, such as SDS/PAGE, immunoblot analysis and densitometry, not specifically mentioned have been described elsewhere [20,26].

RESULTS

Biochemical properties of the recombinant rat TK proteins

Comparison of the predicted TK protein sequences revealed that seven arginine residues (Arg^{55}, Arg^{102}, Arg^{133}, Arg^{350}, Arg^{433}, Arg^{470} and Arg^{506} in rat TK) are highly conserved among several organisms including yeast, rats and humans [15–20]. Four (Arg^{102}, Arg^{350}, Arg^{433} and Arg^{506}) out of the seven conserved arginines were chosen, based on the likely distances and potential number of hydrogen-bonds between each arginine and the highly conserved loop or the TPP binding site, as determined by molecular modelling analysis (see below). Each arginine was replaced by alanine because of alanine's smaller size and lack of net charge. The four mutant proteins as well as the wild-type TK were produced in *E. coli* and their catalytic activities and levels of immunoreactive protein were measured.

The molecular mass of the rat TK monomer (His-tagged) produced in *E. coli* was about 74 kDa (Figure 1A), as judged by denaturing SDS/PAGE. This value was similar to that of TK isolated from mammalian tissues [20,24]. Under our conditions, a similar level of rat TK protein was expressed from each of the expression vectors, as determined by immunoblot analysis using the polyclonal antibody against rat TK. This antibody did not recognize the *E. coli* TK (Figure 1A, lane 2), probably because of the low similarity (< 30 %) between the two proteins [20].

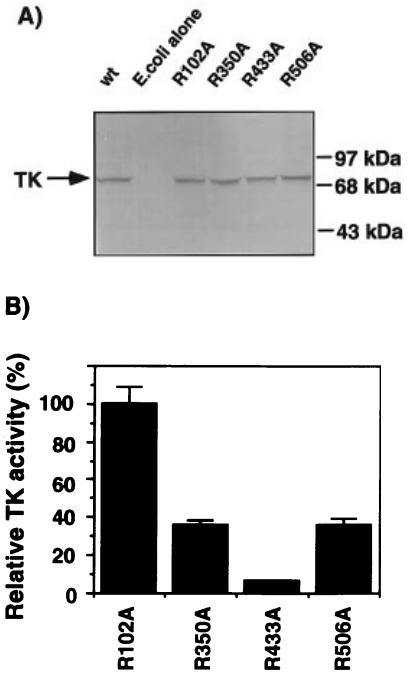

Figure 1 Relative catalytic activities of rat TK mutants produced in *E. coli*

(**A**) Soluble fractions (200 μg/sample) of *E. coli* lysates harbouring cDNAs for the wild-type (wt) and various rat TK mutants as indicated were subjected to 10% SDS/PAGE, followed by immunoblot analysis using polyclonal antibody against rat enzyme [20]. Migration of protein markers (97 kDa, phosphorylase *b*; 68 kDa, BSA; and 43 kDa, ovalbumin) is shown on the right. (**B**) Activities of the recombinant TK proteins were measured by the production of sedoheptulose 7-phosphate [23], based on the density of immunoreactive band (**A**) and Coomassie Blue stained gel (not shown). Each value in (**B**) represents the average of five determinations with S.D.

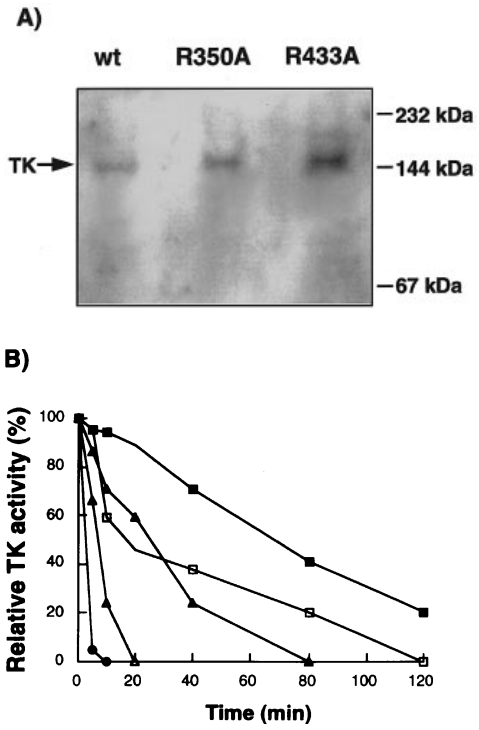

Figure 2 Immunoblot analysis of the wild-type and two mutants of recombinant rat TK

(**A**) Soluble fractions (300 μg/well) of *E. coli* lysates harbouring plasmids for the wild-type (wt, lane 1), $Arg^{350} \rightarrow$ Ala (lane 2), or $Arg^{433} \rightarrow$ Ala (lane 3) proteins were separated on a 10% Tris/glycine non-denaturing polyacrylamide gel and subjected to immunoblot analysis as described in the Experimental section. Migration of protein markers (BSA, 67 kDa; lactate dehydrogenase, 144 kDa; and catalase, 232 kDa) is shown on the right. (**B**) To determine heat sensitivity, the purified TK (wild-type, squares; $Arg^{350} \rightarrow$ Ala, triangles; and $Arg^{433} \rightarrow$ Ala, circles) was incubated at 55 °C in the absence (open symbols) or presence (closed symbols) of 2 mM TPP and 5 mM Mg^{2+}. Aliquots (25 μM of each protein) from the reaction mixtures were taken at the indicated times and assayed for TK activity.

Endogenous TK activity from *E. coli* extract lacking the rat TK cDNA insert was very low under our assay conditions (usually less than 2–3 % of that of the wild-type with the cDNA insert) and was used as a negative control. The $Arg^{102} \rightarrow$ Ala mutant showed the same activity as the wild-type, whereas both $Arg^{350} \rightarrow$ Ala and $Arg^{506} \rightarrow$ Ala mutants exhibited about 37 % activity (Figure 1B) when similar amounts of recombinant proteins (Figure 1A) were used. The latter value is in agreement with the 31 % activity of the wild-type protein, seen with Arg^{359} in yeast TK (equivalent to Arg^{350} of rat enzyme), as recently reported [21]. The greatest decrease in activity level was, however, observed with the $Arg^{433} \rightarrow$ Ala mutant, which showed only 6.1 % of the activity of the wild-type. This suggests that the critical arginine in TK may be the one at position 433.

Owing to similar activities of the $Arg^{350} \rightarrow$ Ala and $Arg^{506} \rightarrow$ Ala mutants (Figure 1), $Arg^{350} \rightarrow$ Ala was chosen for further studies to compare its role with that of Arg^{433} in TK activity. The reduced TK activities of $Arg^{350} \rightarrow$ Ala and $Arg^{433} \rightarrow$ Ala mutants were not likely to be due to the dissociation of the homodimers, since these recombinant TK proteins migrated identically (approx. 148 kDa) on a non-denaturing gel (Figure 2A).

Table 2 Kinetic analyses of the purified wild-type TK and Arg^{433} → Ala mutant

The purified wild-type TK (WT) and Arg^{433} → Ala mutant were used to determine the kinetic parameters by the method described [24]. V_{max} values are expressed as specific activity [μmol NADH oxidized · min^{-1} · (mg of protein)$^{-1}$]. Each value represents the average of three determinations ± S.D. at different substrate concentrations (up to 20 mM) in the presence of 10 mM of the other pairing substrate. The kinetic constants determined once in the presence of 6 mM of the second substrate were similar to those obtained at 10 mM of the second substrate, as shown below.

	Xylulose 5-phosphate			Ribose 5-phosphate		
Enzyme	V_{max}	K_m (μM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	V_{max}	K_m (μM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
WT	4.80 ± 0.42	39.9 ± 4.5	2.9×10^5	2.31 ± 0.30	36.2 ± 3.5	1.5×10^5
Arg^{433} → Ala	0.28 ± 0.03	457.9 ± 47.2	1.5×10^3	0.4 ± 0.05	879.2 ± 85.3	1.1×10^3

Thermostability of purified TK proteins

To determine the stability of the mutant TK proteins, we further purified the three recombinant TK proteins (wild-type, Arg^{350} → Ala, and Arg^{433} → Ala) to near homogeneity using Ni^{2+}-affinity column chromatography. Coomassie Blue staining of an SDS-polyacrylamide gel revealed that each of the TK proteins was more than 99.5 % pure (results not shown). We then studied the thermosensitivity of these purified TK proteins at 55 °C and their protection by the cofactor TPP. Under our experimental conditions, both mutants lost their activities faster than the wild-type TK in the absence and presence of TPP (Figure 2B). The wild-type had an apparent half-life of about 60 min in the presence of 2 mM TPP, whereas its half-life was reduced to 25 min in the absence of TPP. The Arg^{350} → Ala mutant had a half-life of 25 and 7.5 min with and without TPP respectively. These results indicate that TPP markedly protected the wild-type TK and Arg^{350} → Ala mutant from heat inactivation. In contrast, the half-life of the Arg^{433} → Ala mutant was too short to be measured accurately, either with or without TPP (Figure 2B). These results suggest that the Arg^{433} → Ala mutant was much less stable than the wild-type or Arg^{350} → Ala mutant.

Kinetic analyses of purified TK proteins

Since Arg^{433} → Ala exhibited the lowest activity and the least thermostability among the recombinant TK mutants, the kinetic parameters of the wild-type TK and Arg^{433} → Ala mutant were then determined at different substrate concentrations (up to 20 mM) in the presence of 6 or 10 mM of the other pairing substrate. Under these conditions, the catalytic activities of the purified TK proteins seemed to reach maximum levels (Table 2). The apparent V_{max} values of the wild-type and Arg^{433} → Ala mutant were 4.8 and 0.4 μmol NADH oxidized · min^{-1} · (mg of protein)$^{-1}$ respectively. The apparent K_m values for xylulose 5-phosphate of the wild-type and Arg^{433} → Ala mutant were 39.9 and 457.9 μM respectively, whereas those for ribose 5-phosphate were 36.2 and 879.2 μM respectively. The K_m values of the Arg^{433} → Ala mutant for two substrates, xylulose 5-phosphate and ribose 5-phosphate, were 11.5- and 24.3-fold higher respectively than those of the wild-type. The k_{cat}/K_m values of the Arg^{433} → Ala mutant were markedly reduced to less than 1 % of the wild-type TK in the presence of 10 mM of the second substrate (Table 2), suggesting an important role of Arg^{433} in the TK activity.

Molecular modelling of rat TK

The biochemical characterization of the recombinant rat TK proteins was complemented by homology modelling of rat enzyme using the coordinates for the crystal structure of yeast TK [21] as the template. Our homology modelling analysis revealed that there was a high level of structural similarity in the functionally important domains (such as TPP-binding sites and the highly conserved loop). This similarity exists between yeast and rat TK, despite only a 45 % overall sequence similarity [20]. Using our modelled rat TK structure, the potential importance of the seven conserved arginines (Arg^{55}, Arg^{102}, Arg^{133}, Arg^{350}, Arg^{433}, Arg^{470} and Arg^{506}) was assessed by their proximity to the highly conserved loop (amino acids 447–462 in the rat enzyme, comparable with amino acids 468–483 in the yeast counterpart), as well as to the cofactor-binding site [4,27–29]. According to our model (Figure 3A), Arg^{55}, Arg^{102}, and Arg^{470} were located at least 8 Å or more away from the highly conserved loop or the cofactor-binding region. Arg^{133} does not form a hydrogen bond with any of the residues in the highly conserved loop, despite its proximity to it. Arg^{350} and Arg^{506} do not interact with the highly conserved loop on the same or the other monomer, although they do interact with the phosphate group of the substrate erythrose 4-phosphate [21]. Arg^{433} is located at the interface between the two subunits (Figure 3A, at the middle of the groove on the V-shaped monomer). The proximity (within 4.0 Å) of Arg^{433} (monomer A) to the highly conserved loop on the other monomer (monomer B) allows the guanidinium moiety of arginine to interact with the backbone of amide oxygen atoms of Pro^{458}, Ser^{459} and Met^{461}, as well as with the carboxylate oxygen of Glu^{464}, via multiple hydrogen-bonds (Figure 3B). An alanine at position 433 of the Arg^{433} → Ala mutant was incapable of forming any hydrogen-bond with the highly conserved loop in monomer B (Figure 3C). The loss of inter-subunit hydrogen bonds in the Arg^{433} → Ala mutant is most likely the cause of the marked loss (> 90 %) of TK activity. Our results suggest that the distance (less than 4.0 Å) and the hydrogen-bond interaction with the highly conserved loop are two important factors in supporting maximum catalytic activity.

DISCUSSION

Primary sequence comparisons of TKs in various species suggest that these enzymes are evolutionarily conserved in their functional domains, such as the TPP binding site and the catalytically important highly conserved loop [4,15–20]. Despite recent structural analyses [4,21,27–29], the functional role of some other highly conserved amino acids has not been elucidated. For example, the location of the functionally important arginine (one arginine per TK monomer), which Kremer et al. [14] originally reported, is still unknown. To determine the location of this critical arginine and to study the functional roles of conserved arginine residues in rat TK activity, we performed site-directed

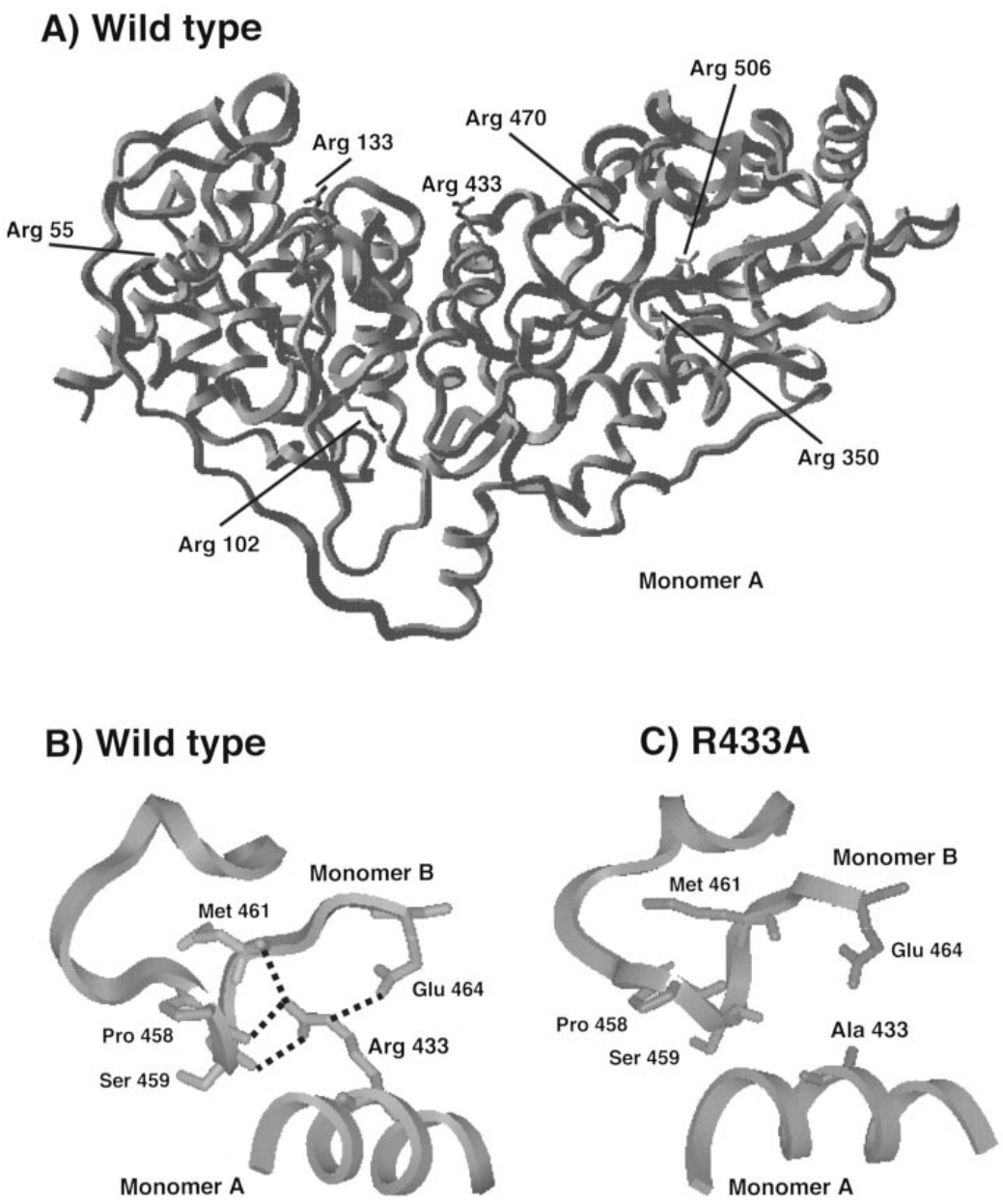

Figure 3 Computer-assisted molecular modelling analysis of rat TK (wild-type and Arg^{433} → Ala mutant)

(**A**) The overall structure of monomer A of rat TK wild-type was performed by homology modelling, based on the coordinates for the crystal structure of yeast TK [21] as template. The highly conserved seven arginine residues in various species [15–20] are also indicated. (**B**) Potential hydrogen-bond interactions between Arg^{433} on monomer A and the amino acids marked in the highly conserved loop shown on monomer B of the wild-type protein are indicated with dotted lines. The δ-nitrogen atom of the guanidinium moiety of Arg^{433} can only form one hydrogen-bond out of the two possible interactions with either Ser^{459} or Glu^{464}. In contrast, no hydrogen-bond interaction can be drawn in the Arg^{433} → Ala mutant (**C**). The whole structure in each case was arbitrarily rotated to provide the best view of the potential hydrogen-bonds.

mutagenesis studies followed by biochemical and structural analyses.

The wild-type and mutant rat TK proteins produced in bacteria were characterized biochemically. Biochemical data suggest that the presence of the extra six histidine residues at the N-terminus and subsequent purification by single-step affinity chromatography did not significantly affect the catalytic activity of the recombinant wild-type TK. Kinetic analysis of the purified wild-type and Arg^{433} → Ala mutant (Table 2) showed that the Arg^{433} → Ala mutant has markedly reduced activity. A contributing factor to this reduced activity may result from the much lower affinities of the mutant for the substrates than the wild-type TK. Considering the established mechanism of a Bi Bi Ping-Pong catalysis by TK and the possible indirect interaction of the critical arginine with the substrates [14], it is conceivable that the changes in the kinetic parameters in the Arg^{433} → Ala mutant were a result of a secondary effect, following potential alteration of the optimal conformation of the highly conserved loop at the subunit interface subsequent to the loss of multiple inter-subunit hydrogen-bond interactions (see below).

Our demonstration of the protective effect of TPP on TK thermostability (Figure 2B) is consistent with earlier data by Jeyasingham et al. [30], who reported that TPP can stabilize TK. The presence of TPP may render TK more stable by forming multiple hydrogen bonds with amino acids on both subunits, as recently indicated [4,21,27–29]. The lack of the inter-subunit hydrogen bonds may contribute to destabilization of the $Arg^{433} \rightarrow$ Ala homodimer at 55 °C faster than the wild-type or $Arg^{350} \rightarrow$ Ala proteins, as observed in the thermosensitivity experiment (Figure 2B). Despite the differences in stability, the replacement of the side-chain at Arg^{433} or Arg^{350} with alanine might not induce gross conformational changes, as indicated by our preliminary CD spectral data (not shown). The importance of Arg^{433} for the possible stabilization of rat TK via inter-subunit interactions is comparable with the functional role in dimer stabilization of Glu^{162} of yeast TK, which is also located at the interface between the two subunits, as recently reported [31].

A recent study has demonstrated that His^{481} in the highly conserved loop of yeast TK is involved in the critical rate-limiting step of the proton abstraction from TPP [28]. Our modelling analysis of rat TK predicted that Arg^{433} could form at least three inter-subunit hydrogen bonds (Figure 3B) with the highly conserved loop which contains Gln^{460} (equivalent to His^{481} of yeast TK). Gln^{460}, conserved in both rat and human [20], can abstract a proton from TPP, as described [32]. Therefore, the disruption of these inter-subunit hydrogen bonds in the $Arg^{433} \rightarrow$ Ala mutant could result in perturbation of the optimal conformation of the highly conserved loop [4,20,28], thus leading to alteration in the catalytically important process of Gln^{460}-mediated proton abstraction in rat TK. This idea is supported by the marked changes in the V_{max}, K_m, and k_{cat}/K_m values of the $Arg^{433} \rightarrow$ Ala mutant, compared with those of the wild-type (Table 2).

To definitely demonstrate that Arg^{433} is the crucial arginine (one per TK monomer), as reported [14], the direct effects of arginine modifying agents on the $Arg^{433} \rightarrow$ Ala or $Arg^{350} \rightarrow$ Ala mutants need to be studied. However, these studies were difficult to perform, mainly due to the extremely low activity of the $Arg^{433} \rightarrow$ Ala mutant ($< 10\%$ of that of the wild-type). Nevertheless, the following arguments strongly suggest that Arg^{433}, rather than Arg^{350} or Arg^{506}, is the catalytically important arginine residue. First, the catalytic activity of the $Arg^{433} \rightarrow$ Ala mutant was much lower than that of the wild-type protein, $Arg^{350} \rightarrow$ Ala or $Arg^{506} \rightarrow$ Ala mutants (Figure 1). Second, both Arg^{350} and Arg^{506} (equivalent to Arg^{359} and Arg^{528} of yeast TK) simultaneously interact with the phosphate group of a sugar phosphate substrate [21]. This fact is inconsistent with the earlier report that the critical arginine residue does not directly serve as the binding site for the substrates [14]. Third, in our modelled rat TK structure, neither Arg^{350} nor Arg^{506} can form intra- or inter-subunit hydrogen bonds with the highly conserved loop, whereas Arg^{433} can form multiple inter-subunit hydrogen bonds (Figure 3). Fourth, the $Arg^{433} \rightarrow$ Ala protein is much less stable than the wild-type or $Arg^{350} \rightarrow$ Ala mutant, probably resulting from the loss of inter-subunit hydrogen bond interactions (Figure 3C). Lastly, the drastic reductions in the k_{cat}/K_m values of the $Arg^{433} \rightarrow$ Ala mutant, relative to those of the wild-type, for both substrates (Table 2), indicate an important role of this residue in the catalysis.

In summary, using site-directed mutagenesis followed by production of the recombinant proteins in *E. coli*, biochemical characterization and molecular modelling, we have demonstrated the critical role of a previously uncharacterized Arg^{433} in TK catalysis. Furthermore, we have demonstrated the probable role of this arginine in the maintenance of the optimal conformation of the catalytically important highly conserved loop.

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